(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00814 A3

(51) International Patent Classification[7]: C12N 15/10, G01N 33/68, C07K 14/00, A61K 38/17

(21) International Application Number: PCT/EP00/05922

(22) International Filing Date: 26 June 2000 (26.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/344,096 25 June 1999 (25.06.1999) US

(71) Applicant *(for all designated States except US)*: UNIVERSITÄT ZÜRICH [CH/CH]; Rämistrasse 71, CH-8006 Zürich (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PLÜCKTHUN, Andreas [DE/CH]; Möhrlistrasse 97, CH-8006 Zürich (CH). ARNDT, Katja [DE/US]; 559 A North Street, Oakland, CA 94609-1201 (US). MÜLLER, Kristian [DE/US]; 559 A North Street, Oakland, CA 94609-1201 (US). PELLETIER, Joelle [CA/CA]; Département de Chimie, Pavillon Principal, Université de Montréal, 2900 Edouard Montpetit, Montréal, Québec H3C 357 (CA).

(74) Agent: VOSSIUS & PARTNER; Siebertstrasse 4, D-81765 München (DE).

(81) Designated States *(national)*: CA, JP, US.

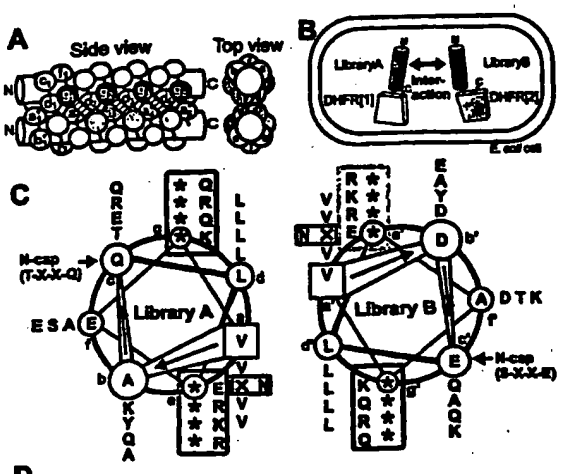
(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

[Continued on next page]

(54) Title: HETERO-ASSOCIATING COILED-COIL PEPTIDES AND SCREENIGN METHOD THEREFOR

(57) Abstract: The present invention relates to methods for the identification of novel hetero-associating coiled-coil peptides and uses of these peptides for hetero-dimerization of fusion proteins. It furthermore relates to vectors, host cells useful for the production of these novel hetero-association peptides and (poly)peptides/proteins comprising these peptides.



D

clone (occurence)	LibraryA e1	r1	e2	r2	e3	r3	e4	r4	LibraryB e'1	r'1	e'2	r'2	e'3	r'3	e'4	r'4
#1 (22x)	E	K	R	Q	N	K	R	R	Q	E	Q	E	N	K	K	R	K
#2 (3x)	Q	K	R	R	N	K	E	E	K	E	E	K	Q	N	K	K	Q
#3 (2x)	E	Q	Q	R	N	K	K	K	K	R	K	Q	E	N	E	E	Q
#4 (1x)	E	R	R	Q	N	K	K	E	Q	E	E	E	Q	N	Q	R	E
#5 (1x)	E	Q	E	E	N	K	K	R	R	K	K	K	K	N	R	K	R
#6 (3x)	Q	Q	E	Q	N	E	E	K	Q	R	Q	Q	K	N	R	R	K
#7 (2x)	E	R	K	E	N	E	E	K	R	Q	E	Q	Q	N	R	Q	K
#8 (1x)	E	K	K	K	N	K	K	K	K	R	Q	E	Q	N	E	Q	E
#9 (1x)	Q	E	Q	E	N	R	E	E	E	Q	K	K	E	N	Q	K	Q
#10 (1x)	R	R	R	R	N	Q	K	K	R	R	E	E	E	N	R	R	R

WO 01/00814 A3

(88) Date of publication of the international search report:
22 March 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/EP 00/05922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 G01N33/68 C07K14/00 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N G01N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 34120 A (PELLETIER JOELLE NINA ;REMY INGRID (CA); UNIV MONTREAL (CA); MICHN) 6 August 1998 (1998-08-06) cited in the application the whole document	1-22
Y	YU Y ET AL: "INVESTIGATION OF ELECTROSTATIC INTERACTIONS IN TWO-STRANDED COILED-COILS THROUGH RESIDUE SHUFFLING" BIOPHYSICAL CHEMISTRY,AMSTERDAM,NL, vol. 59, 16 April 1996 (1996-04-16), pages 299-314, XP000971324 cited in the application the whole document	1-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 January 2001	18/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Smalt, R

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ARNDT K M ET AL: "In-vivo selection of interacting peptide libraries by selectively-infective phages." FASEB JOURNAL, vol. 11, no. 9, 1997, page A1327 XP002156485 17th International Congress of Biochemistry and Molecular Biology in conjunction with the Annual Meeting of the American Society for Biochemistry and Molecular Biology;San Francisco, California, USA; August 24-29, 1997 ISSN: 0892-6638 the whole document	
A	O'SHEA E K ET AL: "PEPTIDE 'VELCRO*': DESIGN OF A HETERODIMERIC COILED COIL" CURRENT BIOLOGY,GB,CURRENT SCIENCE,, vol. 3, no. 10, 1993, pages 658-667, XP000653001 ISSN: 0960-9822 cited in the application the whole document	
A	HODGES R S: "DE NOVO DESIGN OF ALPHA-HELICAL PROTEINS: BASIC RESEARCH TO MEDICALAPPLICATIONS" BIOCHEMISTRY AND CELL BIOLOGY. BIOCHIMIE ET BIOLOGIE CELLULAIRE,XX,XX, vol. 74, no. 2, 1996, pages 133-154, XP000605834 ISSN: 0829-8211 cited in the application the whole document	
A	JOHN MATTHIAS ET AL: "Two pairs of oppositely charged amino acids from Jun and Fos confer heterodimerization to GCN4 leucine zipper." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 23, 1994, pages 16247-16253, XP002156486 ISSN: 0021-9258 cited in the application the whole document	
A	O'SHEA E K ET AL: "MECHANISM OF SPECIFICITY IN THE FOS-JUN ONCOPROTEIN HETERODIMER" CELL, vol. 68, no. 4, 1992, pages 699-708, XP002156487 ISSN: 0092-8674 cited in the application the whole document	

-/—

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PELLETIER, J.N. ET AL.: "An in vivo library-versus-library selection of optimized protein-protein interactions." NATURE BIOTECHNOLOGY, vol. 17, July 1999 (1999-07), pages 683-90, XP002156488 the whole document	1-19,22
P,X	ARNDT KATJA M ET AL: "A heterodimeric coiled-coil peptide pair selected in vivo from a designed library-versus-library ensemble." JOURNAL OF MOLECULAR BIOLOGY, vol. 295, no. 3, 21 January 2000 (2000-01-21), pages 627-639, XP002156489 ISSN: 0022-2836 the whole document	1-19,22

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claim 8, and claims 10-13 and 15-22 in as far as they pertain to claim 8, relate to a polypeptide defined by reference to a desirable characteristic or property, namely that it can be obtained by the method of claim 7.
The claims cover all polypeptides having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such polypeptides. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the polypeptides comprising domains as defined in claims 5 and/or 6, and in the broader sense those that fall under the general fomulae of claim 1(a) and (b).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Interr nal Application No
PCT/EP 00/05922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9834120 A	06-08-1998	CA 2196496 A	31-07-1998
		AU 5850598 A	25-08-1998
		EP 0966685 A	29-12-1999

Wetherell, John R.

From: Kim, Chang H.
Sent: Wednesday, August 14, 2002 10:18 AM
To: Abrams, William F.; Wetherell, John R.
Subject: RE: CBR: OLD to respond to Pharmastem's discovery responses

I talked to Evelyn, and defendants are working towards responding to PharmaStem's discovery requests on 8/21. She will circulate Bio-Cell's responses this week for review. Defendants will follow up with producing documents within 30 days from 8/21. We'll take a look at Bio-Cell's responses before drafting CBR's responses.

-----Original Message-----
From: Abrams, William F.
Sent: Tuesday, August 13, 2002 6:01 PM
To: Kim, Chang H.
Subject: RE: CBR: OLD to respond to Pharmastem's discovery responses

I think so. But let's confirm that position with them first.

William F. Abrams
Pillsbury Winthrop LLP
2550 Hanover Street
Palo Alto, California 94304-1115
Voice: 650-233-4668
Fax: 650-233-4545
Desktop Fax: (866) 741-0216 (please also send hard copy fax to above number)
wabrams@pillsburywinthrop.com
wabrams@stanford.edu

-----Original Message-----
From: Kim, Chang H.
Sent: Tuesday, August 13, 2002 5:38 PM
To: Abrams, William F.
Subject: RE: CBR: OLD to respond to Pharmastem's discovery responses

Should we take StemCyte's position and say CBR will produce on August 28?

-----Original Message-----
From: Abrams, William F.
Sent: Tuesday, August 13, 2002 4:35 PM
To: Kim, Chang H.
Subject: FW: CBR: OLD to respond to Pharmastem's discovery responses

We need to nail down this extension.

William F. Abrams
Pillsbury Winthrop LLP
2550 Hanover Street
Palo Alto, California 94304-1115
Voice: 650-233-4668
Fax: 650-233-4545
Desktop Fax: (866) 741-0216 (please also send hard copy fax to above number)
wabrams@pillsburywinthrop.com

wabrams@stanford.edu

<< Message: CBR: OLD to respond to Pharmastem's discovery responses >>

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/62, 15/13, C07K 15/28 — A1

(11) International Publication Number: WO 93/15210

(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/EP93/00082

(22) International Filing Date: 15 January 1993 (15.01.93)

(30) Priority data:
92101069.0 23 January 1992 (23.01.92) EP
(34) Countries for which the regional or international application was filed: AT et al.

(71) Applicant *(for all designated States except US)*: MERCK PATENT GMBH [DE/DE]; Frankfurter Str. 250, Postbox 4119, D-6100 Darmstadt (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : PLÜCKTHUN, Andreas [DE/DE]; Jägerwirtstr. 3, D-8000 München 70 (DE). PACK, Peter [DE/DE]; Wilhelm-Busch-Str. 5/6, D-8000 München 71 (DE).

(81) Designated States: AU, CA, HU, JP, KR, NO, PL, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MONOMERIC AND DIMERIC ANTIBODY-FRAGMENT FUSION PROTEINS

(57) Abstract

The present invention describes a new class of antigen binding molecules which contain Fv-fragments of an antibody but do not use the constant antibody domains. They can also dimerize with other antibody fragment molecules or with non-antibody fragment molecules to form bi- or multifunctional antibody-fragment fusion proteins and so-called miniantibodies, respectively. The new fusion proteins can be used in the broad field of diagnostic and therapeutical medicine.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

Monomeric and Dimeric Antibody-Fragment Fusion Proteins

The present invention describes a new class of antigen binding molecules which contain Fv-fragments of an antibody but do not use the constant antibody domains. They can also dimerize with other antibody fragment molecules or with non-antibody fragment molecules to form bi- or multifunctional antibody-fragment fusion proteins and so-called mininantibodies, respectively. The new fusion proteins can be used in the broad field of diagnostic and therapeutical medicine.

Background of the invention

Since a few years there is a great interest in the biotechnological field to modify naturally occuring antibodies in order to obtain more specified and more individual antibody species. Therefore, attempts have been made to produce (modified) antibody fragments.

All naturally occuring antibodies of all classes have at least two binding sites. This enables them to bind to a surface with a greater functional affinity (also called avidity) than monovalent fragments, such as Fab fragments. Over the last few years, methods have been described (Skerra and Plückthun, 1988, Science 240, 1038-1040 ; Better et al., 1988, Science 240, 1041-1043) with which

functional antibody fragments can be produced in Escherichia coli. These include the Fv fragment (the heterodimer consisting of V_H and V_L) and the Fab fragment (consisting of the complete light chain with the domains V_L and C_L as well as the first two domains of the heavy chains V_H and C_{H1}).

The Fv fragment, however, has a tendency to dissociate into V_H and V_L and therefore, it is advantageous to link the two domains covalently. One particular way of linking them is by designing a peptide linker between them, either in the orientation V_H-linker-V_L or V_L-linker-V_H (Bird et al.,1988, Science 242, 423; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85, 5879) The resulting fragments are called single-chain Fv fragments.

All these fragments are, however, monovalent. We describe in this invention a method to engineer small dimerization domains based on peptides forming amphipathic helices. These peptides will be referred to as "intercalating", but this term is only meant to express the ability of targeted association and not a restriction referring to a particular structure of the dimerization interface.

While the methodology described here, is in principle applicable to either Fab, Fv or scFv fragments, it is the latter for which their use is most advantageous. In this case bivalent fragments can be constructed of very small size, and still the dissociation into V_L and V_H as well as the wrong matching of fragment chains, e.g. V_L-V_L, can be prevented.

Antibody fragments of small size are of particular advantage in many applications. In diagnostic applications (e. g. ELISA, RIA, etc.), the smaller molecules surface decreases the problems of nonspecific interactions, which are known to frequently involve the constant domains. The same is true in using antibody fragments as ligands in affinity chromatography. In tumor diagnostics or therapy, it is important that a significant proportion of the injected antibody penetrates tissues and localizes to the tumor, and is dependend on the molecular dimensions (Colcher et al., 1990, J. Natl. Cancer Inst. 82, 1191-1197). Expression yields and secretion efficiency of recombinant proteins are also a function of chain size (Skerra & Plückthun, 1991, Protein Eng. 4, 971) and smaller proteins are preferred for this reason. Therefore, molecules of a small size are advantageous for several reasons.

Previously, decreasing the molecular dimensions of the antibody meant the preparation of proteolytic fragments. The smallest bivalent fragments, $(Fab)'_2$ fragments, are still about twice the size of the present fragments of this invention. Therefore, these new fragments combine three features: (a) small size, (b) bivalence or bifunctionality and (c) ability of functional expression in E. coli.

There is great interest in bifunctional antibodies in a large number of areas. Bifunctional antibodies may be defined as having two different specificities for either two different antigens or for two epitopes of the same antigen.

There are currently a number of methods how to produce bifunctional antibodies. However, none of the existing methods allows to produce exclusively bifunctional antibodies in vivo, but rather a mixture of molecular species always occur, requiring complicated and expensive separation procedures.

Four principal methods can be distinguished. In the first, chemical crosslinking is used, which may advantageously use heterobifunctional crosslinkers. By this method, whole antibodies (Staerz et al., 1985, Nature 314, 628; Perez et al, 1985, Nature 316, 354-356), Fab fragments (Carter et al., 1992, Biotechnology 10, 163) and scFv fragments (Cumber et al., 1992, J. Immunol. 149, 120) have been chemically crosslinked after purification.

The second previous method involved the fusion of two hybridomas to give a so-called heterohybridoma or "quadroma". In this method, any light chain can pair with any heavy chain, and the two heavy chains can give homodimers or heterodimers resulting in very complicated product mixtures (Milstein & Cuello, 1983, Nature 305, 537).

The third method is related to the second and consists of transfecting two expression plasmids into a hybridoma cell, encoding the heavy and light chain of the second antibody (Lenz & Weidle, 1990, Gene 87, 213) or a retroviral vector (De Monte et al., 1990, Acad. Sci. 87, 2941-2945). However, once introduced, the product mixture is identical as in the second procedure.

Finally, antibodies have been reduced, mixed and reoxidized (Staerz & Bevan, 1986, Immunology Today 7). Again, very complicated product mixtures are obtained requiring sophistical separation and quality control procedures.

Thus a method is still needed allowing the isolation of exclusively heterodimeric antibodies directly without the complicated preparation required from chemical crosslinking. In the present invention, this problem is solved by (i) covalently linking corresponding VH and VL domain in a scFv fragment and (ii) using dimerization domains only allowing the formation of heterodimers, such as certain leucine zippers and derivatives.

Another important consideration in the present invention was the desire to make the MW of the bispecific antibody as small as possible for reasons explained above in detail. This was achieved by using scFv fragments.

A number of uses of bispecific antibodies bave been described, and most of them would benefit from this new technology. For example, bispecific antibodies are of great interest in tumor therapy. One arm of the antibody may bind to a tumor marker, the other arm to a T-cell epitope, a toxin, or a radionuclide binding peptide or protein to bring a killing function close to the tumor cell. In diagnostics, one arm may bind to the analyte of interest and the other to a principle which can easily be quantified, e. g. an enzyme. Finally, in cellular applications, it may be advantageous to obtain higher selectivity in binding, if two different epitopes or the same protein complex can be recognized or if two different proteins can be recognized on the same cell surface.

Thus, it was object of the invention to create new individual and stable antibody fragment fusion proteins with bi- or even mulitfunctional binding sites.

It has been found that antibody fragment fusion proteins containing Fv-fragments could be produced by genetic engineering methods which show specified and improved properties.

Object of the invention is, therefore, a monomeric antibody-fragment fusion protein essentially consisting of a Fv-fragment of an antibody and a peptide which is capable to dimerize with another peptide by noncovalent interaction.

The term "noncovalent interaction" means every existing under normal condititions stable linkage which is not related to a covalent binding, for example linkage by Van der Waal's forces, (steric) interdigitation of amphiphilic peptides, especially peptide helices, or peptides bearing opposite charges of amino acid residues. The correspondingly effective peptides are called above and below interactive or intercalating peptides.

The amphiphilic peptides consist of up to 50 amino acids. Preferrably they consist of 10 to 30 amino acids. In a preferred embodiment of the invention the interactive peptide is a peptide helix bundle (comprising of a helix, a turn and another helix, see above). In another embodiment the interactive peptide is a leucine zipper consisting of a peptide having several repeating amino acids, in which every seventh amino acid is a leucine residue. In other cases according to the invention the peptide bear positively or negatively

charged residues, e.g. lysine (positively charged) or glutamic acid (negatively charged) in a way that this peptide can bind to another peptide (of a second monomeric unit) bearing opposite charges.

The Fv-fragment and the intercalating peptide are linked together either directly or by a linker peptide, preferrably by a linker peptide. In a preferred embodiment the linker peptide is a hinge region sequence of an antibody.

As defined, the Fv-fragment consists of the V_L and V_H region of an antibody. The Fv-fragment according to the invention is preferrably a single chain fragment. Single chain fragments can be obtained by standard techniques using standard linker molecules.

Furthermore, object of the invention is a dimeric fusion protein essentially consisting of two monomeric fusion proteins, wherein the linkage of the monomeric units bases on noncovalent interaction of identical or different peptides, characterized in that at least one monomeric unit is an antibody-Fv-fragment fusion protein as defined above.

If the dimer contains two Fv-fragments, the Fv-fragments may be the same (identical antigen binding sites) or may be different (different antigen binding sites). In these cases mono- and bispecific (Fv)- miniantibodies can be obtained. According to the invention bispecific mini-antibodies are preferred.

The interactive peptides may be the same or may be different; preferrably, they are identical. The intercalating peptides may be associated in parallel or in antiparallel fashion.

Object of the invention is, therefore, above all, a dimeric fusion protein consisting of two Fv-fragments with different specificity (antigen binding sites) and identical intercalating helix peptides, the antibody fragments and the peptides are linked together by a hinge region sequence.

Furthermore, object of the invention is a dimer consisting of a monomeric unit containing a Fv-fragment and another monomeric unit wherein the Fv-Fragment was replaced by a non-antibody peptide. The non-antibody peptide may be a toxin, like ricin, a chelator- or metal binding peptide, or an enzyme (e.g. marker enzyme), or a peptide bearing a detectable lable (e.g. a radioisotope).

The non-antibody peptide can also bear a corresponding binding site for said groups, binding sites directed to T-cells or T-cell fragments included.

Furthermore, the invention relates to monomers and dimers, as defined above, wherein the interactive peptide(s) is (are) additionally fused at the C-terminus to target proteins/peptides as mentioned above, the corresponding binding sites included. Thus, the resulting fusion proteins and miniantibodies, respectively, are multifunctional.

The invention relates, furthermore, to a process for preparation of a monomeric antibody fusion protein as defined above, characterized in that the genes coding for the Fv-fragment, the interactive peptide and, if desired, the linking peptide are cloned into one expression plasmid, a host cell is transformed with said expression plasmid and cultivated in a nutrient solution, and the monomeric fusion protein is expressed in the cell or secreted into the medium.

Object of the invention is, finally, a process for preparation of a dimeric fusion protein as defined above, characterized in that the genes coding for the complete monomeric fusion proteins or parts of it are cloned at least into one expression plasmid, a host cell is transformed with said expression plasmid(s) and cultivated in a nutrient solution, and either the complete dimeric fusion protein is expressed in the cell or into the medium, or the monomeric fusion proteins are separately expressed and the noncovalent linkage between the two monomeric units is performed in the medium or in vitro, and in the case that only parts of the fusion proteins were cloned, protein engineering steps are additionally performed according to standard techniques.

The dimeric Fv-fragments containing fusion proteins according to the invention show a high avidity against corresponding antigens and a satisfying stability. These novel bivalent or bifunctional molecules can be prepared as folded and assembled molecules in E. coli. These miniantibodies are compatible with functional expression by secretion.

Detailed description of the invention

The oligomerization domains were selected for having a fairly small molecular weight and for being compatible with transport of the fusion protein through the membrane. They are based on two different types of amphiphilic helices.

Amphiphilic helices are known to predominantly, but not exclusively, associate in two different molecular structures: Four helix bundles and coiled coils. The design and formation of helix bundles has been studied previously (Eisenberg et al., 1986, Proteins 1, 16-22; Ho and deGrado, 1987, J. Am. Chem. Soc. 109, 6751-6758; Regan and deGrado, 1988, Science 241, 976-978 ; Hill et al., 1990, Science 294, 543-546). This molecule association is also known from natural proteins (Richardson, 1981, Adv. Prot. Chem. 34, 167).

The four helix bundle may be formed from either four separate molecules (each contributing one helix), two molecules containing two helices each (connected as helix-turn-helix) or one molecule containing a helix-turn-helix-turn-helix-turn-helix motif. For dimerization or multimerization, only the first two are suitable.

Three variations of this latter theme were tested. In the first, one helix of the sequence given in Eisenberg et al. (1986) (Proteins 1, 16-22) was used. In the second, this sequence was extended by a small hydrophilic peptide ending in a cysteine. Once the helices are associated, the hydrophilic peptides are held in sufficiently close contact that they

can collide and a disulfide bond can form under oxidizing conditions, as in the periplasm of E. coli. In the third variation, two helices are used in tandem, separated by a short turn encoding peptide.

In the second design, peptides are used which can form so-called coiled-coil structures. Such peptides occur in transcription factors such as e. g. GCN4 from yeast and have been called leucine zippers (Landschulz et al., 1988, Science 240, 1759-1764). The crystal structure of this has been solved recently (O'Shea et al., 1991, Science 254, 539-544) and showed a parallel arrangement of the helices.

A covalent attachment of the helices is possible by a small peptide extension, again containing a cystein. Since the helices are now parallel, the peptide extension can be much shorter, since the distance is much smaller.

The various dimerization devices (intercalating helices) were however not fused to the antibody domain directly. It is advantageous to introduce a flexible peptide between the end of the scFv fragment and the beginning of the helix. As an example, the upper hinge region of mouse IgG3 has been used. However, a variety of hinges can be used. It is not required for dimerization per se, but provides a spacing of the two scFv domains similar to the antigen binding sites of a whole antibody. This way, the two binding sites span a greater distance in space and therefore can reach neighboring antigens on a solid surface.

The naturally occuring hinges of antibodies are preferred embodiments of hinges in bivalent miniantibodies. In the case of bifunctional miniantibodies, the hinges may be shorter, since frequently molecules from different surfaces are to be crosslinked as close as possible, and flexibilty of the dimer is not necessary. The choice of the hinge is governed by the desired residue sequence,l ength (Argos, 1990, J. Mol. Biol. 211, 943-958), compatibility with folding and stability of the amphiphilic helices (Richardson & Richardson, 1988, Science 240, 1648-1652), secretion and resistance against proteases .

The present invention deals with peptides as dimerization devices, which should be as small as possible. One preferred embodiment is the use of peptides which can form amphipathic helices. Such helices shield the hydrophobic surface by dimerization or even multimerization. Helices of this type are characterized by their having hydrophobic patches on one face of the helix, and containing a sufficient number of helix-forming residues. Rules for such peptides are discussed in Eisenberg et al. 1986, O'Shea et al.,.1991 (Science 254, 539-544),1992 (Cell 68, 699-708).

Natural peptides of this type are found as the so-called leucine zippers, characterized by a periodic occurence of leucine (every seventh residue) and other hydrophilic residues (e. g. valine) also every seventh residue. As these priniples are now understood (O'Shea et al. 1991, 1992, literature cited), the sequence can be varied to incorporate

residues which make the association of homodimers unfavorable, but favors the association of heterodimers. Such sequence alteration can e. g. involve the incorporation of charge bridges, such that in the homodimers, like charges repel each other and in the heterodimer, opposite charges attract each other (see below).

The present invention can also be extended to bifunctional miniantibodies. In this case, dimerization devices (intercalation peptides) have to be used which will only allow the formation of heterodimers, but not homodimers. A preferred embodiment of this part of the invention are two different coiled-coil helices, such as in naturally occuring leucine zippers, e. g. from the transcription factor proteins jun and fos (O'Shea et al.,1989, Science 245, 646-648).

In a further embodiment of the invention, the constant scFv-hinge-helix can be extended at the C-terminus to result in a fusion protein. For example, a fusion to an enzyme may be made to use such bivalent constructs in diagnostics. Such enzymes are e. g. alkaline phosphatase, luciferase or horse radish peroxidase. The advantage of such a antibody-enzyme fusion protein would be that the bivalence of the antibody would lead to an enhanced binding to the surface-bound antigen. The advantage over a fusion protein prepared by conventional technology (i. e. chemical coupling of the antibody to the enzyme of choice) would be a greater batch-to-batch consistency; homogeneity of the product and the much simpler method of preparation, namely from E. coli in a single step.

In the same fashion, the miniantibodies may be extended at the C-terminus to incorporate a toxin. Such immunotoxins would be bivalent or even bispecific and thus combine the advantages of such antibody fragments linked above with the advantages in tumor therapy known for immunotoxins. Similarly, a metal binding peptide or protein could be linked genetically to be used in radioimmunotherapy or in tumor imaging. The same advantages for any genetically encoded hybrid protein hold true as given above for the antibody-enzyme fusions.

In another embodiment of the invention, a construct of the type scFv-hinge-helix may be made to dimerize with another protein fused to a dimerization domain, in complete analogy as described above for the formation of bispecific miniantibodies. In this fashion, the scFv fragment would e. g. be fitted with the helix of the fos protein. Such foreign protein, which could be made to form heterodimers with the scFv fragment, include enzymes useful in diagnosis, toxins, metal-binding peptides or proteins useful in radioimmunotherapy or radio-imaging.

Using the principles of this invention, the dimerization domains presented here can also serve for purification purposes. A recombinant protein of any kind can be fused to a dimerization domain, e.g. to hinge-fos-zipper. After coexpression with a scFv-hinge-jun, the heterodimer can be purified in one step with an affinity column for the scFv-specificity. In an alternative approach, the 'opposite' zipper, linked to a column support, 'catches' the protein-hinge-zipper when passing through the column as a crude cell extract.

The elution of the pure fusion protein from the column is possible using the unfolding temperature of the zipper. A subsequent separation from the dimerization domain is achievable by introduction of a proteolytic site, e.g. for blood clotting factor Xa, into the hinge (Nagai & Thogerson, 1987, Meth. Enyzmol. 152,,461-481).

A particular advantage of the miniantibodies described in this invention is the ability to assemble functionally in Escherichia coli. In the case of homobivalent constructs, a dimerization principle is used which allows the formation of homodimers. Examples described above include the coiled-coil helix (leucine zipper) of the yeast protein GCN4 or the helices from an antiparallel 4-helix bundle. In this case, the scFv fragment is expressed in the presence of a bacterial signal sequence and carries at the end of the gene of the scFv fragment the codons for a hinge and the dimerization helix or helix-turn-helix. The helices are compatible with secretion to the periplasmic space in E. coli, where protein folding, disulfide formation and assembly occurs. Under these conditions, the homodimeric proteins form by themselves and can directly be isolated in the dimeric form.

If heterobivalent constructs are desired, two different scFv fragments or one scFv fragment associating with a different protein need to associate. In the preferred embodiment of this invention, both proteins to be assembled are expressed in the same cell, preferably on the same plasmid, preferably as a dicistronic operon. The design of artificial dicistronic

operons is explained e.g. in Skerra et al. (1991, Protein Eng. 4, 971). Since the assembly must take place in the periplasm, because the scFv fragment can only fold in the oxidizing milieu, both proteins must be transported and both must be fitted with a signal sequence. The dimerization peptides must be chosen such that they promote the association of two different proteins, but prevent the association of the respective homodimers. Examples of such proteins are the leucine zipper peptides of the proteins fos and jun (see above).

When not expressed in the same cell, the different scFv-hinge-zipper constructs have to be mixed together as a crude cell extract or purified protein and treated with raised temperature. In absence of the 'opposite' zipper, e.g. a scFv-hinge-jun-zipper construct is able to form homodimers. After short heating to the melting temperature of around 40-C, the zippers of the unwanted homodimer unfold and form a much more stable heterodimer (O'Shea et al., 1992, Cell 68, 699-708). Without raising the temperature, formation of heterodimers in vitro is not possible, as tested in experiments.

Short Description of the Figures and the Sequence Listing

Fig. 1 scFv-Expression vector pLISC-SE containg the scFv-fragment.

Fig. 2 Dicistronic scFv-hinge-zipper expression vector pACKxFyJ.

Fig. 3 Functional ELISA;
The concentrations of the affinity purified proteins, measured by OD_{280} (vertical axis), refer to the molar number of binding sites per well (horizontal axis). The ELISA plates were coated with phosphocholine-BSA, and the purified phosphocholine-specific miniantibody-proteins were bound and detected by an anti-McPC603 antiserum.
(a) Comparison of various miniantibodies.
(b) Comparison of miniantibody scHLXc with ScFV and whole IgA.

Fig. 4 Functional Anti-lysozyme ELISA;
PC-affinity purified samples of coexpressed anti-PC-anti-lysozyme bispecific miniantibody. + and - on the horizontal axis means: plus inhibitor (+) and without inhibitor (-).

The attached sequence listing refers to sequence identity numbers (S.I.N.):

S.I.N. 1: Whole nucleotid- and amino acid sequence of the pLISC-SE vector.

S.I.N. 2: Gene cassette of intercalating GCN4-leucine zipper (nucleotid- and amino acid sequence).

S.I.N. 3: Gene cassette encoding intercalating antiparallel helix-turn-helix (nucleotid- and amino acid sequence).

S.I.N. 4: Gene cassette encoding intercalating jun-zipper and IgG3-hinge region.

S.I.N. 5: Gene cassette encoding intercalating fos-zipper and IgG3-hinge region.

S.I.N. 6: Gene cassette encoding intercalating jun-zipper and designed linker.

S.I.N. 7: Gene cassette encoding intercalating fos-zipper and designed linker.

Example 1: Construction of vectors for secreted single-chain fragments, containing a restriction site for introducing genes for intercalating peptides.

Recombinant DNA-techniques were based on Sambrook et al. (1989, Molecular Cloning: A laboratory manual. Second edition. Cold Spring Harbor Laboratory, New York). Functional expression of the single-chain Fv fragments and the minianTibodies in E. coli JM83 was carried out with vectors similar to pASK-lisc (Skerra et al., 1991, Protein Eng. 4, 971). Site directed mutagenesis was directly performed in these vectors according to Kunkel et al. (1987, Meth. Enzymol. 154, 367-382) and Geisselsoder et al. (1987, Biotechniques 5, 786-791) using the helper phage M13K07 (Vieira & Messing, 1987, Meth. Enzymol. 153, 3-11). SDS-PAGE was carried out as described by Fling and Gregerson (1986, Anal. Biochem. 155, 83-88). Concentrations of affinity-purified proteins were measured by OD_{280} using calculated extinction

coefficients (Gill & von Hippel, 1989, Anal. Biochem. 182, 319-326).A vector such as pASK40 (Skerra et al., 1991, Protein Eng. 4, 971) is used, which contains an origin of replication, a regulatable promotor, a bacterial signal sequence followed by a multiple cloning site, a transcription terminator and an origin for single stranded phages.The gene for the single-chain Fv fragment is designed as follows: The nucleotide sequence of a V_H domain is directly followed by a linker sequence encoding preferably about 15 residues, preferably of the sequence $(Gly_4Ser)_3$, followed directly by the sequence of the V_L domain. Alternatively, the sequence of the V_L domain may be directly followed by the sequence of the linker, followed by the sequence of the V_H domain.

If the antibody is of known sequence, the complete gene of the scFv fragment may be assembled from synthetic oligonucleotides. A detailed experimental procedure for such a gene synthesis of an antibody gene is e.g. given in Plückthun et al. (1987, Cold Spring Harbor Symp. Quant. Biol. 52, 105-112).

If the genes of the V_H and V_L domains are present in other vectors, the gene for the scFv fragment may be assembled from restriction fragments. For example, a restriction fragment encoding most of the V_H domain may be excised from another plasmid, and a fragment encoding most of the V_L domain may be excised from a plasmid. The remaining pieces of V_L and V_H and

the linker for the scFv fragment can be provided by cassettes of synthetic oligonucleotides, which need to be ligated by standard methodology (Sambrook et al., 1989, literature cited). The mixture of fragments is ligated into the vector pASK40 or a similar plasmid containing a pair of suitable restriction sites.

If the genes of the antibody have not been cloned before, they may be directly obtained from the hybridoma cell producing the antibody by the polymerase chain reaction (PCR; PCR methodology is described in McPherson et al., 1991, PCR-A Practical Approach Oxford University Press, New York). Primers suitable for amplification of V_H and V_L domains have been given by Orlandi et al., 1989, Proc. Natl. Acad. Sci. USA 86, 3833-3837; Huse et al., 1989, Science 246, 1275-1281; Larrick et al., 1989, Bio-technology 7, 934-938. The methodology of obtaining mRNA from hybridoma is described in these references as well. The separate V_H and V_L genes may be cloned into separate vectors, and the scFv gene assembled according to the principles explained above.

If the ligated fragments do not result in a correct reading frame of the scFv fragment, a precise fusion with the signal sequence codons resident on the plasmid may be generated by site directed mutagenesis. The design of the oligonucleotides and the execution is possible for anyone skilled in the art.

The scFv expression plasmid so obtained contains the codons for a bacterial signal sequence, directly followed by the first variable domain (V_H or V_L), a linker and the second variable domain (V_L or V_H) under the control of a regulatable promotor.

At the 3' end of this genes, corresponding to the C-terminus of the scFv protein, a unique restriction site is introduced into the expression plasmid to allow insertion of cassettes coding for the intercalating peptides. The restriction site is introduced by site directed mutagenesis using the method of Kunkel (1987, Meth. Enzymol. 154, 367-382).

An example of the complete sequence of a suitable single-chain Fv expression plasmid pLISC-SE for receiving an intercalation peptide is shown in Fig. 1 and Sequence Identity No. (S.I.N.) 1 (see Squence Listing).

Example 2: Design and construction of a gene cassette encoding intercalating peptides of a leucine zipper.

The gene cassette, fitted with restriction sites to be compatible with the restriction site at the 3' end of the scFv fragment gene, must encode the sequence of a hinge (connection the scFv fragment to the intercalating peptide) and the intercalation peptide itself. The hinge region, may however also be obmitted.

As an example the sequence of the upper hinge region of mouse IgG3 (Dangl et al., 1988, EMBO J. 7, 1989-1994), followed by the sequence of the leucine zipper sequence of the yeast protein GCN4 (Oas et al., 1990, Biochemistry T29, 2891-2894),

is back-translated into frequently used E. coli codons (S.I.N.: 2). Oligonucleotides are synthesized, and ligated into the vector pLISC-SE, previously digested with EcoRI and Hind III.

Example 3: Design and construction of a gene cassette encoding intercalating peptides of a four-helix bundle.

Analogous to Example 2, the sequence of the upper hinge region of mouse IgG3, followed by the sequence of the helix-turn-helix of a four helix bundle (Eisenberg et al., 1986, literature cited) is backtranslated into frequently used E. coli codons (S.I.N.: 3). Oligonucleotides are synthesized, and ligated into the vector pLISC-SE, previously digested with Eco RI and Hind III.

Example 4: Design and construction two gene cassettes encoding intercalating peptides of a leucine zipper and their co-expression.

Analogous to Example 2, the sequence of the upper hinge region of mouse IgG3 followed by the sequence of the zipper sequence of the jun protein (O'Shea et al., 1992, literature cited), is backtranslated into frequently used E. coli codons (S.I.N.: 4). Oligonucleotides are synthesized, and ligated into the vector pLISC-SE, previously digested with EcoRI and Hind III.

In a parallel reaction, the sequence of the upper hinge region of mouse IgG3, followed by the sequence of the zipper sequence of the fos protein (O'Shea et al., 1992, Cell 68, 699-708), is backtranslated into frequently used E. coli codons (S.I.N.: 5). Oligonucleotides are synthesized, and ligated into the vector pLISC-SE, previously digested with Eco RI and Hind III.The two vectors thus each code for a different antibody scFv fragment, followed by a hinge peptide and a different leucine zipper peptide. To co-express the two scFv fragments, the whole scFv-hinge-zipper gene of the fos-containing product is excised from the vector as a Xba I-Hind III fragment and ligated into the vector, pLISC-SE-scFv-jun, containing already the scFv gene of the other antibody.

The newly obtained vector then expresses the $scFv_1$-$linker_1$-fos-zipper and the $scFv_2$-$linker_2$-jun-zipper from a single promoter as a dicistronic operon.

An improved sequence for the hinge region in the context of fos and jun zippers is given in S.I.N.: 6 and 7. This hinge is shorter and therefore not as susceptible to proteolysis. In cases, where the distance between the two binding sites is of less importance, such shortened hinges may be advantageous. In this case, the "tail" of the scFv fragment has been shortened and the EcoRI site, which receive the genes for the intercalating peptides has been moved four residues upstream.

Example 5: Purification of bivalent miniantibody from E. coli.

E. coli JM83, harboring a plasmid constructed as in examples II and III, are grown to an O.D. 550 of 0.5 and induced with IPTG at a final concentration of 1 mM. The cells are centrifuged, resuspended in BBS buffer (200 mM Na-borate, 160 mM NaCl, pH 8.0) and the suspension is passed through a French press. In these examples, a phosphorylcholine binding miniantibody is used. The miniantibody is purified by passage over a phosphorylcholine affinity chromatography as described (Chesebro and Metzger, 1972, Biochemistry 11, 766-771).

Example 6: Purification of a bispecific miniantibody from E. coli

E. coli JM83, harboring a plasmid constructed as in examples II and III and containing a dicistronic structural gene for two different scFv (Fig. 2), are grown to an O.D. 550 of 0.5 and induced with IPTG at a final concentration of 1mM. The cells are centrifuged, resuspended in BBS buffer (200 mM Na-borate, 160 mM NaCl, pH 8.0) and the suspension is passed through a French press.

In this example, a bispecific miniantibody is used containing both a specificity for phosphorylcholine as well as benzoylampicillin. The miniantibody is purified by passage over a phosphorylcholine affinity chromatography as described (Chesebro and Metzger, 1972, literature cited).

Example 7: Surface binding of bivalent miniantibodies

The ELISA-plates (Nunc, Macrosorp) were coated with 400 -g/ml phosphocholine-BSA in PBS buffer (20 mM phosphate, pH 7.2, 115 mM NaCl). The hapten reagent was prepared from nitrophenyl phosphocholine (Sigma), which was reduced and diazotized essentially as described (Chesebro & Metzger, 1972, literature cited), and reacted by azo-coupling to BSA (Sigma) in borate-saline buffer (52.5 mM sodium borate, pH 9, 120 mM NaCl) at 4-C for 48 hours with subsequent dialysis against PBS. After blocking the non-coated plate surface with 5% skim milk (Nestle) in PBS buffer for at least 2 hours, the periplasmic extract or the purified protein was incubated in BBS buffer on the plate for 90 min at room temperature. After thorough washing (3 times), remaining functional antibody fragments were detected according standard procedures (Harlow & Lane, 1988, "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory, 555-592) with rabbit anti-McPC603 serum and anti-rabbit immunoglobulin linked to peroxidase (Sigma) according to Gallati (1979, Clin. Chem. Clin. Biochem. 17, 1-4).

An enormous gain in binding, and thus sensitivity, is observed for all miniantibody constructs, compared to the monomeric scFv fragment. This is consistent with the simultaneous binding of two or even more binding sites to the same surface. _These avidity of the fusion protein scHLXc was comparable to the natural antibody McPC603, which could be detected with antigen-coated ELISA, while the monomeric scFv fragment could only be detected with hundred-fold higher concentrations (Fig. 3 a, b). All binding is nearly totally

inhibitible with soluble hapten, except of the monomeric scFv fragment. The thermodynamic affinity of the natural antibody to soluble phosphocholine is about 1.6 $ 10^5 M^{-1} and thus relatively weak (Metzger et al., 1971, Proceedings of the I st Congress of Imunology. Academic Press, New York, pp. 253-267), and this is apparently not sufficient for a monomeric fragment-hapten complex to survive the repeated washing steps of a functional ELISA (Kemeny & Challacombe, 1988, "ELISA and other solid phase immunoassays", Wiley & Sons, New York).

Example 8: Surface binding of bifunctional miniantibodies

Coexpressed bifunctional miniantibodies recognizing phosphorylcholine with one arm and lysozyme with the other arm were purified by phosphocholine (PC) affinity chromatography and tested for lysozyme specificity. An ELISA-plate was coated with lysozyme, the ELISA was carried out as described in Example VII.Three different preparations show binding to the antigen-surface, which is completely inhibitible with soluble lysozyme (Fig. 4).

SEQUENCE LISTING:

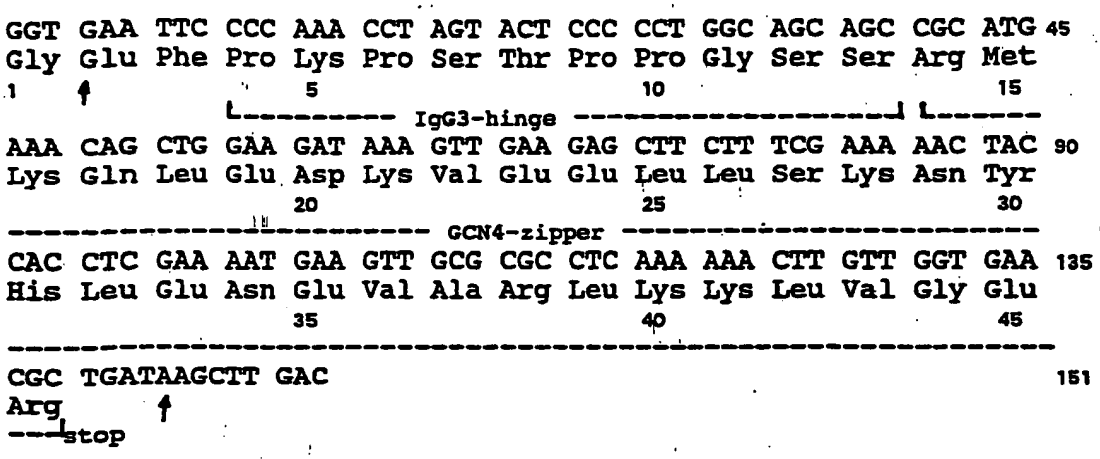
SEQ ID NO: 1

```
ACCCGACACC ATCGAATGGC GCAAAACCTT TCGCGGTATG GCATGATAGC      50
GCCCGGAAGA GAGTCAATTC AGGGTGGTGA ATGTGAAACC AGTAACGTTA     100
TACGATGTCG CAGAGTATGC CGGTGTCTCT TATCAGACCG TTTCCCGCGT     150
GGTGAACCAG GCCAGCCACG TTTCTGCGAA AACGCGGGAA AAAGTGGAAG     200
CGGCGATGGC GGAGCTGAAT TACATTCCCA ACCGCGTGGC ACAACAACTG     250
GCGGGCAAAC AGTCGTTGCT GATTGGCGTT GCCACCTCCA GTCTGGCCCT     300
GCACGCGCCG TCGCAAATTG TCGCGGCGAT TAAATCTCGC GCCGATCAAC     350
TGGGTGCCAG CTGTGTGGTG TCGATGGTAG AACGAAGCGG CGTCGAAGCC     400
TGTAAAGCGG CGGTGCACAA TCTTCTCGCG CAACGCGTCA GTGGGCTGAT     450
CATTAACTAT CCGCTGGATG ACCAGGATGC CATTGCTGTG GAAGCTGCCT     500
GCACTAATGT TCCGGCGTTA TTTCTTGATG TCTCTGACCA GACACCCATC     550
AACAGTATTA TTTTCTCCCA TGAAGACGGT ACGCGACTGG GCGTGGAGCA     600
TCTGGTCGCA TTGGGTCACC AGCAAATCGC GCTGTTAGCG GGCCCATTAA     650
GTTCTGTCTC GGCGCGTCTG CGTCTGGCTG GCTGGCATAA ATATCTCACT     700
CGCAATCAAA TTCAGCCGAT AGCGGAACGG GAAGGCGACT GGAGTGCCAT     750
GTCCGGTTTT CAACAAACCA TGCAAATGCT GAATGAGGGC ATCGTTCCCA     800
CZGCGATGCT GGTTGCCAAC GATCAGATGG CGCTGGGCGC AATGCGCGCC     850
ATTACCGAGT CCGGGCTGCG CGTTGGTGCG GATGTCTCGG TAGTGGGATA     900
CGCAGATACC GAAGACAGCT CATGTTATAT CCCGCCGTTA ACCACCATCA     950
AACAGGATTT TCGCCTGCTG GGGCAAACCA GCGTGGACCG CTTGCTGCAA    1000
CTCTCTCAGG GCCAGGCGGT GAAGGGCAAT CAGCTGTTGC CCGTCTCACT    1050
GGTGAAAAGA AAAACCACCC TGGCGCCCAA TACGCAAACC GCCTCTCCCC    1100
GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG    1150
GAAAGCGGGC AGTGAGCGCA ACGCAATTAA TGTGAGTTAG CTCACTCATT    1200
AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT GTTGTGTGGA    1250
ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACCATGATTA    1300
CGAATTTCTA GATAACGAGG GCAAAAA---ATG AAA AAG ACA GCT ATC   1345
                                 Met Lys Lys Thr Ala Ile
                                  1               5

GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG   1387
Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln
            10                  15                  20

GCC GAA GTT AAA CTG GTA GAG TCT GGT GGT GGT CTG GTA CAG   1429
Ala Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln
        25                  30

CCG GGT GGA TCC CTG CGT CTG TCT TGC GCT ACC TCA GGT TTC   1471
Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Phe
 35                  40                  45

ACC TTC TCT GAC TTC TAC ATG GAG TGG GTA CGT CAG CCC CCG   1513
Thr Phe Ser Asp Phe Tyr Met Glu Trp Val Arg Gln Pro Pro
     50                  55                  60

GGT AAA CGT CTC GAG TGG ATC GCA GCT AGC CGT AAC AAA GGT   1555
Gly Lys Arg Leu Glu Trp Ile Ala Ala Ser Arg Asn Lys Gly
 65                  70                  75

AAC AAG TAT ACC ACC GAA TAC AGC GCT TCT GTT AAA GGT CGT   1597
Asn Lys Tyr Thr Thr Glu Tyr Ser Ala Ser Val Lys Gly Arg
            80                  85                  90
```

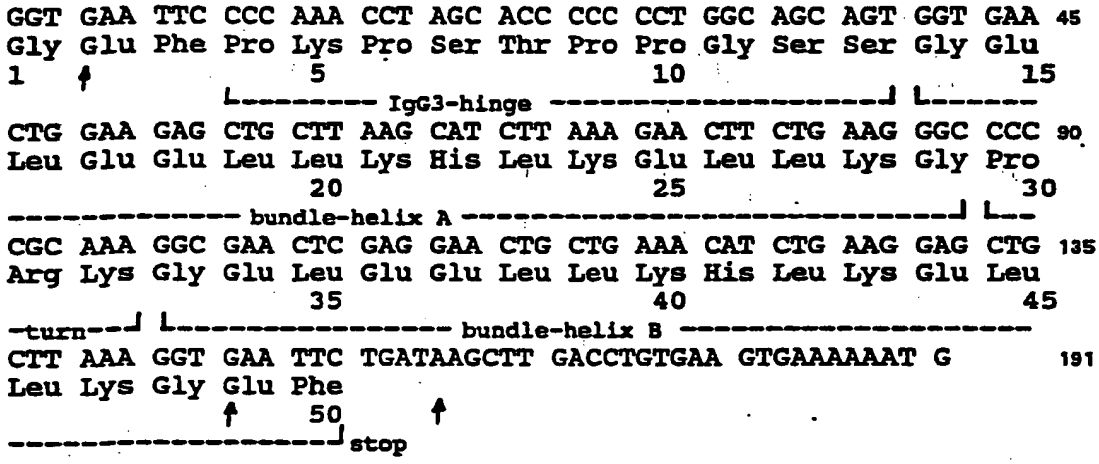
```
TTC ATC GTT TCT CGT GAC ACT AGT CAA TCG ATC CTG TAC CTG      1639
Phe Ile Val Ser Arg Asp Thr Ser Gln Ser Ile Leu Tyr Leu
                95                  100
CAG ATG AAT GCA TTG CGT GCT GAA GAC ACC GCT ATC TAC TAC      1681
Gln Met Asn Ala Leu Arg Ala Glu Asp Thr Ala Ile Tyr Tyr
105                 110                 115
TGC GCG CGT AAC TAC TAT GGC AGC ACT TGG TAC TTC GAC GTT      1723
Cys Ala Arg Asn Tyr Tyr Gly Ser Thr Trp Tyr Phe Asp Val
    120                 125                 130
TGG GGT GCA GGT ACC ACC GTT ACC GTT TCT TCT GGT GGT GGT      1765
Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
        135                 140                 145
GGT TCT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GAT ATC      1807
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile
            150                 155                 160
GTT ATG ACC CAG TCT CCG AGC TCT CTG TCT GTA TCT GCA GGT      1849
Val Met Thr Gln Ser Pro Ser Ser Leu Ser Val Ser Ala Gly
                165                 170
GAA CGT GTT ACC ATG TCT TGC AAA TCT TCT CAG TCT CTG CTG      1891
Glu Arg Val Thr Met Ser Cys Lys Ser Ser Gln Ser Leu Leu
175                 180                 185
AAC TCT GGT AAC CAG AAA AAC TTC CTG GCG TGG TAT CAG CAA      1933
Asn Ser Gly Asn Gln Lys Asn Phe Leu Ala Trp Tyr Gln Gln
    190                 195                 200
AAG CCT GGC CAA CCG CCG AAA CTG CTG ATC TAC GGT GCG TCG      1975
Lsy Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser
        205                 210                 215
ACC CGT GAA TCT GGT GTT CCG GAC CGT TTT ACC GGT AGC GGT      2017
Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
            220                 225                 230
AGC GGT ACC GAC TTC ACT CTG ACC ATC TCT TCT GTA CAG GCT      2059
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala
                235                 240
GAA GAT CTG GCT GTT TAC TAC TGT CAA AAC GAC CAC TCT TAC      2101
Glu Asp Leu Ala Val Tyr Tyr Cys Gln Asn Asp His Ser Tyr
245                 250                 255
CCG CTG ACC TTT GGC GCC GGC ACC AAA CTG GAA CTG AAG CGC      2143
Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
    260                 265                 270
GCT AAC GGT GAA TTC-TGATAAGCTTGA CCTGTGAAGT GAAAAATGGC      2190
Ala Asn Gly Glu Phe *
        275

GCACATTGTG CGACATTTTT TTTGTCTGCC GTTTACCGCT ACTGCGTCAC      2240
GGATCCCCAC GCGCCCTGTA GCGGCGCATT AAGCGCGGCG GGTGTGGTGG      2290
TTACGCGCAG CGTGACCGCT ACACTTGCCA GCGCCCTAGC GCCCGCTCCT      2340
TTCGCTTTCT TCCCTTCCTT TCTCGCCACG TTCGCCGGCT TTCCCCGTCA      2390
AGCTCTAAAT CGGGGCATCC CTTTAGGGTT CCGATTTAGT GCTTTACGGC      2440
ACCTCGACCC CAAAAAACTT GATTAGGGTG ATGGTTCACG TAGTGGGCCA      2490
TCGCCCTGAT AGACGGTTTT TCGCCCTTTG ACGTTGGAGT CCACGTTCTT      2540
TAATAGTGGA CTCTTGTTCC AAACTGGAAC AACACTCAAC CCTATCTCGG      2590
TCTATTCTTT TGATTTATAA GGGATTTTGC CGATTTCGGC CTATTGGTTA      2640
```

```
AAAAATGAGC TGATTTAACA AAAATTTAAC GCGAATTTTA ACAAAATATT 2690
AACGTTTACA ATTTCAGGTG GCACTTTTCG GGGAAATGTG CGCGGAACCC 2740
CTATTTGTTT ATTTTTCTAA ATACATTCAA ATATGTATCC GCTCATGAGA 2790
CAATAACCCT GATAAATGCT TCAATAATAT TGAAAAAGGA AGAGTATGAG 2840
TATTCAACAT TTCCGTGTCG CCCTTATTCC CTTTTTTGCG GCATTTTGCC 2890
TTCCTGTTTT TGCTCACCCA GAAACGCTGG TGAAAGTAAA AGATGCTGAA 2940
GATCAGTTGG GTGCACGAGT GGGTTACATC GAACTGGATC TCAACAGCGG 2990
TAAGATCCTT GAGAGTTTTC GCCCCGAAGA ACGTTTTCCA ATGATGAGCA 3040
CTTTTAAAGT TCTGCTATGT GGCGCGGTAT TATCCCGTAT TGACGCCGGG 3090
CAAGAGCAAC TCGGTCGCCG CATACACTAT TCTCAGAATG ACTTGGTTGA 3140
GTACTCACCA GTCACAGAAA AGCATCTTAC GGATGGCATG ACAGTAAGAG 3190
AATTATGCAG TGCTGCCATA ACCATGAGTG ATAACACTGC GGCCAACTTA 3240
CTTCTGACAA CGATCGGAGG ACCGAAGGAG CTAACCGCTT TTTTGCACAA 3290
CATGGGGGAT CATGTAACTC GCCTTGATCG TTGGGAACCG GAGCTGAATG 3340
AAGCCATACC AAACGACGAG CGTGACACCA CGATGCCTGT AGCAATGGCA 3390
ACAACGTTGC GCAAACTATT AACTGGCGAA CTACTTACTC TAGCTTCCCG 3440
GCAACAATTA ATAGACTGGA TGGAGGCGGA TAAAGTTGCA GGACCACTTC 3490
TGCGCTCGGC CCTTCCGGCT GGCTGGTTTA TTGCTGATAA ATCTGGAGCC 3540
GGTGAGCGTG GGTCTCGCGG TATCATTGCA GCACTGGGGC CAGATGGTAA 3590
GCCCTCCCGT ATCGTAGTTA TCTACACGAC GGGGAGTCAG GCAACTATGG 3640
ATGAACGAAA TAGACAGATC GCTGAGATAG GTGCCTCACT GATTAAGCAT 3690
TGGTAACTGT CAGACCAAGT TTACTCATAT ATACTTTAGA TTGATTTAAA 3740
ACTTCATTTT TAATTTAAAA GGATCTAGGT GAAGATCCTT TTTGATAATC 3790
TCATGACCAA AATCCCTTAA CGTGAGTTTT CGTTCCACTG AGCGTCAGAC 3840
CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT TTCTGCGCGT 3890
AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG GTGGTTTGTT 3940
TGCCGGATCA AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC 3990
AGAGCGCAGA TACCAAATAC TGTCCTTCTA GTGTAGCCGT AGTTAGGCCA 4040
CCACTTCAAG AACTCTGTAG CACCGCCTAC ATACCTCGCT CTGCTAATCC 4090
TGTTACCAGT GGCTGCTGCC AGTGGCGATA AGTCGTGTCT TACCGGGTTG 4140
GACTCAAGAC GATAGTTACC GGATAAGGCG CAGCGGTCGG GCTGAACGGG 4190
GGGTTCGTGC ACACAGCCCA GCTTGGAGCG AACGACCTAC ACCGAACTGA 4240
GATACCTACA GCGTGAGCTA TGAGAAAGCG CCACGCTTCC CGAAGGGAGA 4290
AAGGCGGACA GGTATCCGGT AAGCGGCAGG GTCGGAACAG GAGAGCGCAC 4340
GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA TCTTTATAGT CCTGTCGGGT 4390
TTCGCCACCT CTGACTTGAG CGTCGATTTT TGTGATGCTC GTCAGGGGGG 4440
CGGAGCCTAT GGAAAAACGC CAGCAACGCG GCCTTTTTAC GGTTCCTGGC 4490
CTTTTGCTGG CCTTTTGCTC ACATG 4515
```

SEQ ID NO: 2



SEQ ID NO: 3



SEQ ID NO: 4

```
GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CGT ATC 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Ile
1   ↑           5                  10                  15
            └------IgG3-hinge----------------------┘ └------
GCT CGT CTC GAG GAA AAA GTT AAA ACC CTG AAA GCT CAG AAC TCC 90
Ala Arg Leu Glu Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser
                20                  25                  30
---------------------- jun-zipper ----------------------
GAA CTG GCT TCC ACC GCT AAC ATG CTG CGT GAA CAG GTT GCT CAG 135
Glu Leu Ala Ser Thr Ala Asn Met Leu Arg Glu Gln Val Ala Gln
                35                  40                  45
---------------------- jun-zipper ----------------------
CTG AAA CAG AAA GTT ATG AAC TAC TGATAAGCTT GACCTGTGAA G     180
Leu Lys Gln Lys Val Met Asn Tyr
                50              ↑
------------------------------┘ stop
```

SEQ ID NO: 5

```
GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CTG ACC 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Leu Thr
1   ↑           5                  10                  15
            └---------- IgG3-hinge ----------┘ └------
GAC ACC CTG CAG GCT GAA ACC GAC CAG CTG GAA GAC AAA AAA TCC 90
Asp Thr Leu Gln Ala Glu Thr Asp Gln Leu Glu Asp Lys Lys Ser
                20                  25                  30
---------------------- fos-zipper ----------------------
GCT CTG CAG ACC GAA ATC GCT AAC CTG CTG AAA GAA AAA GAA AAA 135
Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys
                35                  40                  45
---------------------- fos-zipper ----------------------
CTG GAA TTT ATC CTG GCT GCT TAC TGATAAGCTT GACCTGTGAA G     180
Leu Glu Phe Ile Leu Ala Ala Tyr
                50              ↑
------------------------------┘ stop
```

SEQ ID NO: 6

```
GGT GAA TTC CCG TCT GGT AAC GAA GCT CGT ATC GCT CGT CTC GAG  45
Gly Glu Phe Pro Ser Gly Asn Glu Ala Arg Ile Ala Arg Leu Glu
1   ↑           5                  10                  15
                └---- linker ------┘ └-------------------
GAA AAA GTT AAA ACC CTG AAA GCT CAG AAC TCC GAA CTG GCT TCC  90
Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser Glu Leu Ala Ser
                  20                  25                  30
------------------------ jun-zipper ----------------------
ACC GCT AAC ATG CTG CGT GAA CAG GTT GCT CAG CTG AAA CAG AAA  135
Thr Ala Asn Met Leu Arg Glu Gln Val Ala Gln Leu Lys Gln Lys
                  35                  40                  45
------------------------ jun-zipper ----------------------
GTT ATG AAC TAC TGATAAGCTT GACCTGTGAA GTGAAAAATG GCG         180
Val Met Asn Tyr     ↑
---------------┘ stop
```

SEQ ID NO: 7

```
GGT GAA TTC GGT CCG TCT GGT AAC GAA CTG ACC GAC ACC CTG CAG  45
Gly Glu Phe Gly Pro Ser Gly Asn Glu Leu Thr Asp Thr Leu Gln
1   ↑           5                  10                  15
            └--- linker ----------┘ └------ fos-zipper --------
GCT GAA ACC GAC CAG CTG GAA GAC AAA AAA TCC GCT CTG CAG ACC  90
Ala Glu Thr Asp Gln Leu Glu Asp Lys Lys Ser Ala Leu Gln Thr
                  20                  25                  30
-------------------- fos-zipper ------------------------------
GAA ATC GCT AAC CTG CTG AAA GAA AAA GAA AAA CTG GAA TTT ATC  135
Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile
                  35                  40                  45
-------------------- fos-zipper ------------------------------
CTG GCT GCT TAC TGATAAGCTT GACCTGTGAA GTGAAAAATGGCG          180
Leu Ala Ala Tyr     ↑
---------------┘ stop
```

Patent claims

1. Monomeric antibody-fragment fusion protein essentially consisting of a Fv-fragment of an antibody and a peptide which is capable to dimerize with another peptide by noncovalent interaction.

2. Monomer according to claim 1 characterized in that the Fv-fragment is a single chain fragment.

3. Monomer according to claim 1 or 2 characterized in that the interactive peptide consists of 10 to 50, preferrably 10 to 30 amino acids.

4. Monomer according to one of the claims 1 or 3 characterized in that the peptide consists of at least one helix.

5. Monomer according to claim 4 characterized in that the helix peptide consists of a helix, a turn and another helix.

6. Monomer according to claims 4 characterized in that the peptide contains a leucine zipper molecule, having several repeating amino acids, in which every seventh amino acid is a leucine.

7. Monomer according to claims 4 characterized in that the peptide bears charged residues.

8. Monomer according one of the claims 1 to 7 characterized in that a linking peptide is between the Fv-fragment and the peptide.

9. Monomer according to claim 8 characterized in that the linking peptide is a hinge region sequence of an antibody or a fragment thereof.

10. Process for preparation of a monomeric antibody fusion protein as defined in claims 1 to 9, characterized in that the genes coding for the Fv-fragment, the interactive peptide and, if desired, the linking peptide are cloned into one expression plasmid, a host cell is transformed with said expression plasmid and cultivated in a nutrient solution, and the monomeric fusion protein is expressed in the cell or secreted into the medium.

11. Process according to claim 10 characterized in that the host cell is E. coli.

12. Dimeric fusion protein essentially consisting of two monomeric fusion proteins, wherein the linkage of the monomeric units bases on noncovalent interaction of identical or different peptides, characterized in that at least one monomeric unit is an antibody-fragment fusion protein as defined in claims 1 to 9.

13. Dimeric fusion protein according to claim 12 wherein the interactive peptides are the same.

14. Dimer according to claim 12 or 13 characterized in that the second monomeric unit is an antibody-fragment fusion protein as defined in claims 1 to 9 having different specificity.

15. Dimer according to claim 12 or 13 characterized in that the second monomeric unit is a fusion protein as defined in claims 1 to 9, wherein the antibody-fragment (Fv) is replaced by a non-antibody protein or peptide.

16. Dimer according to claim 15 characterized in that the protein or peptide is a toxin, a chelator peptide, a metal binding protein or an enzyme, or has the corresponding specific binding site.

17. Dimer according to claim 15 characterized in that the protein or peptide has a T-cell-, or a T-cell fragment specific binding site.

18. Dimer according to one of the claims 12 to 17, wherein another protein is fused at the C-terminus of one or both of the intercalating peptides.

19. Dimer according to claim 18, wherein the fused protein is a toxin, a chelator peptide, a metal binding protein or an enzyme, or has the corresponding specific binding site, or has a T-cell (fragment) specific binding site.

20. Process for preparation of a dimeric fusion proteinas defined in claims 12 to 19 characterized in that the genes coding for the complete monomeric fusion proteins or parts of it are cloned at least into one expression plasmid, a host cell is transformed with said expression plasmid(s) and cultivated in a nutrient solution, and either the complete dimeric fusion protein is expressed in the cell or into the medium, or the monomeric fusion proteins are separately expressed and the noncovalent linkage between the two monomeric units is performed in the medium or in vitro, and in the case that only parts of the fusion proteins were cloned protein engineering steps are additionally performed.

21. Process according to claim 20 characterized in that the gene coding for the first monomeric fusion protein is cloned into a first expression plasmid, and the gene coding for the second monomeric fusion protein is cloned into a second expression plasmid.

22. Process according to claim 20 characterized in that the noncovalent linkage between the monomeric units forming the dimeric fusion protein is performed in vitro.

23. Process according to one of the claims 20 to 22 characterized in that the host cell is E. coli.

24. Construction kit for preparation of seletive dimers of antibody-fragment fusion proteins as defined in claims 12 to 19 containing(a) a monomeric antibody-fragment fusion protein as defined in claims 1 to 8, and (b) a second monomeric fusion protein as defined in (a), wherein the antibody fragment has the same or another antigen specificity, or wherein the antibody fragment unit is replaced by a non-antibody protein/peptide.

FIG. 1

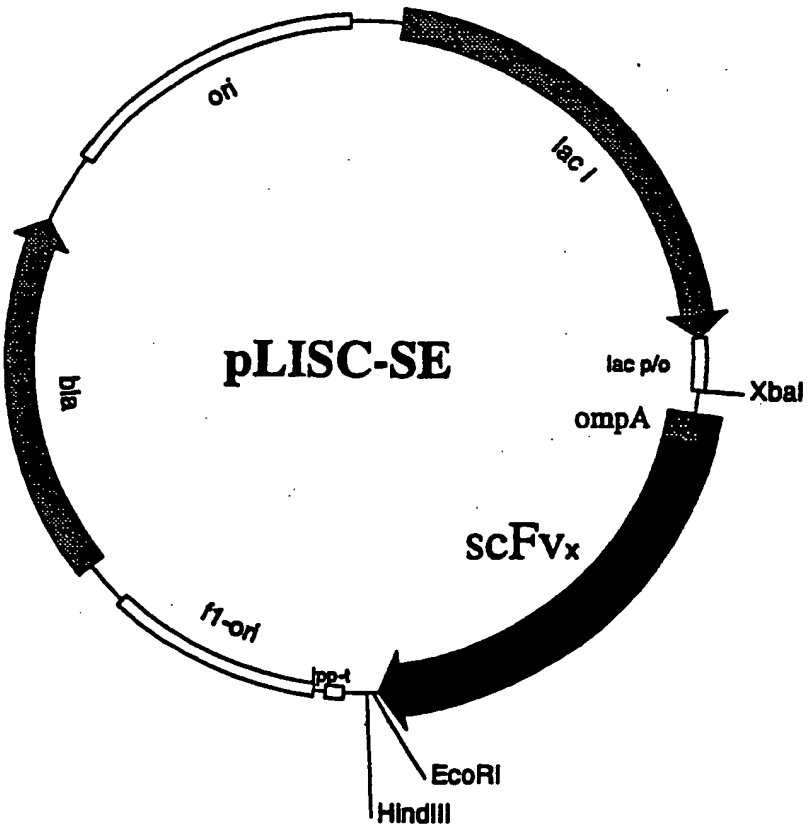

pLISC-SE
ori
lac I
lac p/o
XbaI
ompA
scFvx
f1-ori
lpp-t
EcoRI
HindIII
bla

Fig. 2

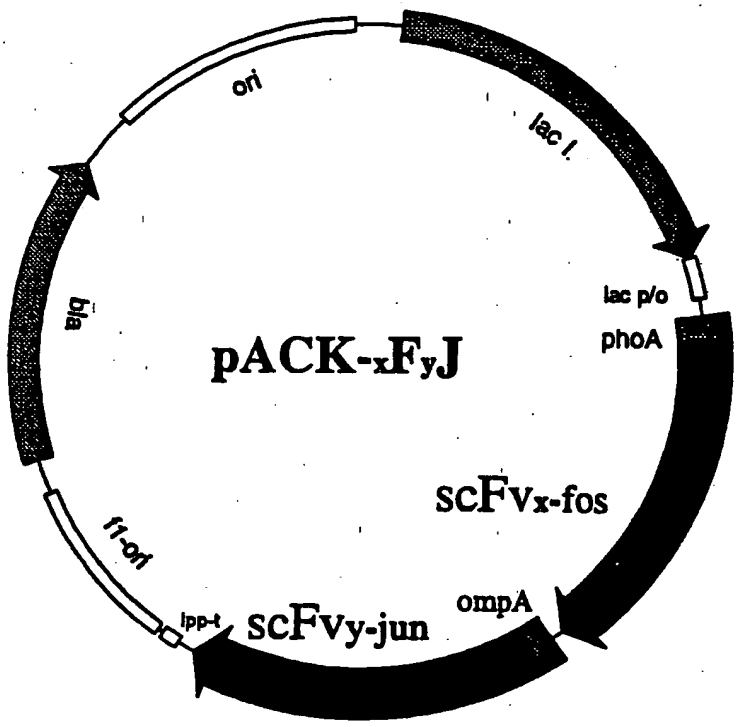

ori
lac I
lac p/o
phoA
pACK-xFyJ
bla
scFvx-fos
f1-ori
lpp-t
scFvy-jun
ompA

Fig. 3 a

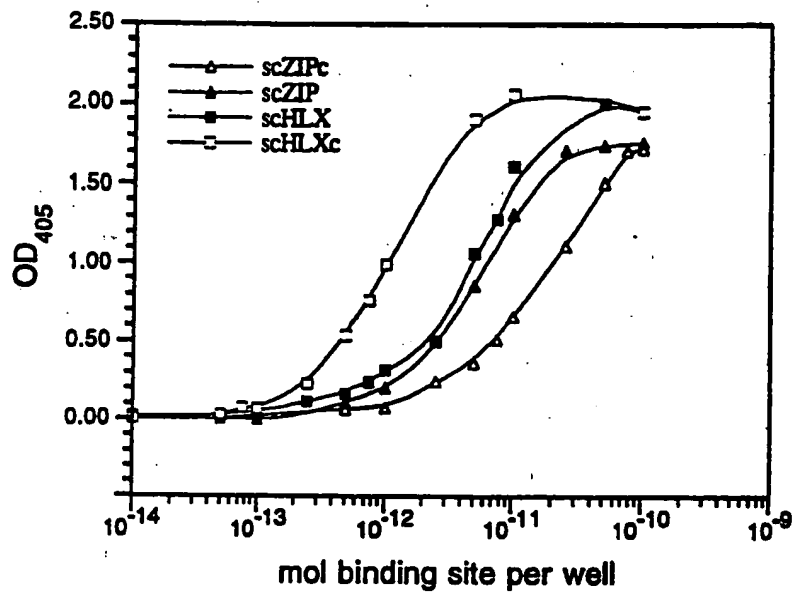

scZIPc
scZIP
scHLX
scHLXc
OD405
2.50
2.00
1.50
1.00
0.50
0.00
10-14
10-13
10-12
10-11
10-10
10-9
mol binding site per well

Fig. 3 b

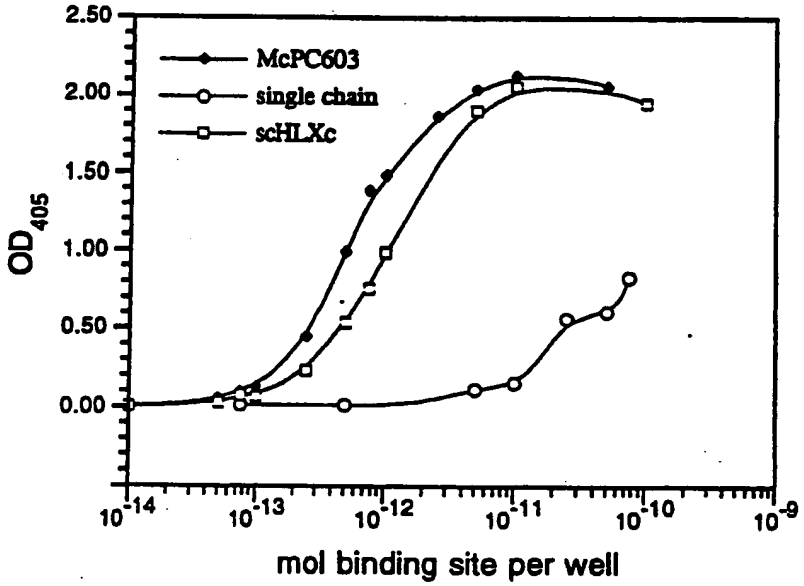
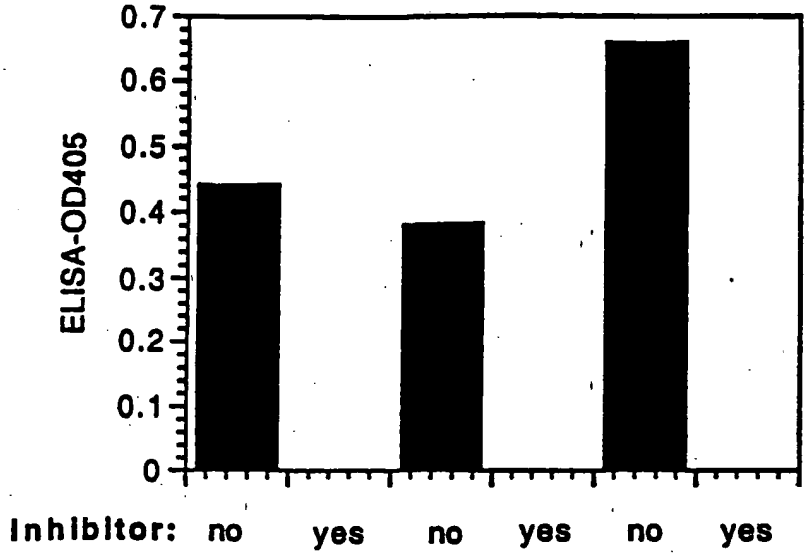

McPC603
single chain
scHLXc
OD405
2.50
2.00
1.50
1.00
0.50
0.00
10-14
10-13
10-12
10-11
10-10
10-9
mol binding site per well

Fig. 4



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/00082

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/62; C12N15/13; C07K15/28

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	TRENDS IN BIOTECHNOLOGY vol. 9, April 1991, CAMBRIDGE GB pages 132 - 137 BIRD AND WALKER 'Single chain antibody variable regions' see page 136, column 3, paragraph 2	1-7, 10-24
Y	PROTEIN ENGINEERING vol. 4, no. 4, April 1991, ENGLAND GB pages 457 - 461 BLONDEL AND BEDOUELLE 'Engineering the quarternary structure of an exported protein with a leucine zipper' see page 461, column 1, last paragraph	1-7, 10-24

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 JUNE 1993	21 -06- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CUPIDO M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOCHEMISTRY vol. 31, no. 6, 18 February 1992, EASTON, PA US pages 1579 - 1584 PACK AND PLÜCKTHUN 'Miniantibodies: Use of amphipatic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in Escherichia coli' see the whole document	1-24

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.